REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
08/16/2013	FINAL	05/15/2010-05/14/2013

4. TITLE AND SUBTITLE

Engineering Ultrastable Protein Filaments into 2D and 3D Templates for Advanced Nanomaterials: a New Dimension in Materials Design

5a. CONTRACT NUMBER
FA 9550-10-1-0214

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Sponsored Projects Office
2150 Shattuck Avenue, Suite #300
Berkeley, CA 94704-5940

10. SPONSOR/MONITOR'S ACRONYM(S)
AFOSR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
FA 9550-10-1-0214

12. DISTRIBUTION / AVAILABILITY STATEMENT
The report is approved for public release/distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this project we endeavoured to expand the realm of multidimensional protein assembly by generating new proteins that assemble into 2D and 3D shapes of controllable size and symmetry, for template-based construction of advanced biomaterials. We successfully created a standardized biomolecular construction kit comprised of a thermostable filamentous protein called gamma-prefoldin and complementary protein "parts" that can be assembled into diverse structures of controllable size and shape. (see report submission for the remainder of the abstract)

15. SUBJECT TERMS

Protein engineering, nanobiotechnology, self-assembling, archaea, thermostable, nanomaterials.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU		Douglas S. Clark
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AFOSR Final Project Report - August 2013

Engineering Ultrastable Protein Filaments into 2D and 3D Templates for Advanced Nanomaterials: a New Dimension in Materials Design

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Future nanoscale device fabrication will increasingly rely on the bottom-up assembly of complex architectures to position functional molecules in regular patterns with nanometer precision. In this regard, proteins are particularly promising because they possess inherent molecular recognition and self-assembly capabilities that produce intricate nanoscale structures with genetically encoded functionalities [1]. We endeavoured to expand the realm of multidimensional protein assembly by generating new proteins that assemble into 2D and 3D shapes of controllable size and symmetry, for template-based construction of advanced biomaterials. One of the central protein building blocks that enabled this effort is the γ-prefoldin (γ-PFD), a chaperone protein isolated from the hyperthermophilic archaeon *Methanocaldococcus jannaschii* [2]. The γ-PFD oligomerizes into filaments that grow up to several microns in length and exhibit remarkable thermostability up to at least 94°C for their quaternary structure [3]. The γ-PFD filament is comprised of monomeric subunits that assemble into filaments through association domains composed of β-sheets, with the remainder of the protein protruding downwards in coiled-coil domains that create a brush-like structure (Fig. 1A). The malleable and distinct modular nature of the γ-PFD filament provided an ideal starting point to construct novel protein architectures for nanobiotechnology applications. Importantly, the interface between each γ-PFD subunits is pliant and amenable to structural modification to control the overall filament length [4]; the interface and the extended coiled-coils of the subunits are physically separate and can be modified independently for expanded function [5]; and the assembled protein is extremely stable to thermal and solvent-induced denaturation [2,3]. Initial studies aimed at engineering γ-PFD filaments used rational design to create a capping protein, called TERM, to control the length of the filament [4]. *The overall aim of this project was to develop a standardized biomolecular "Tinkertoy kit" construction set comprised the γ-PFD and complementary protein "parts" from other sources that can be used to construct a wide range of 2D and 3D protein structures. These higher-order protein structures will have a broad range of applications including as templates for nanostructured molecular machines and bio-inorganic materials.*

The primary objectives of the proposed research were as follows:

1. Combine powerful high-throughput selection techniques with protein shape heuristics to create new 2D and 3D protein architectures of tailorable dimensions. This approach will be generally applicable for the design of robust protein shapes suitable for conjugation to form functional materials.

2. Use new functionalized protein constructs as 2D and 3D templates for the development of nanostructured bio-inorganic materials.

3. Develop protocols for expanding the versatility and processability of the γ-PFD and its constructs, including solubilization in organic solvents, controlled orientation on solid surfaces, and enhanced stability to extreme temperatures.

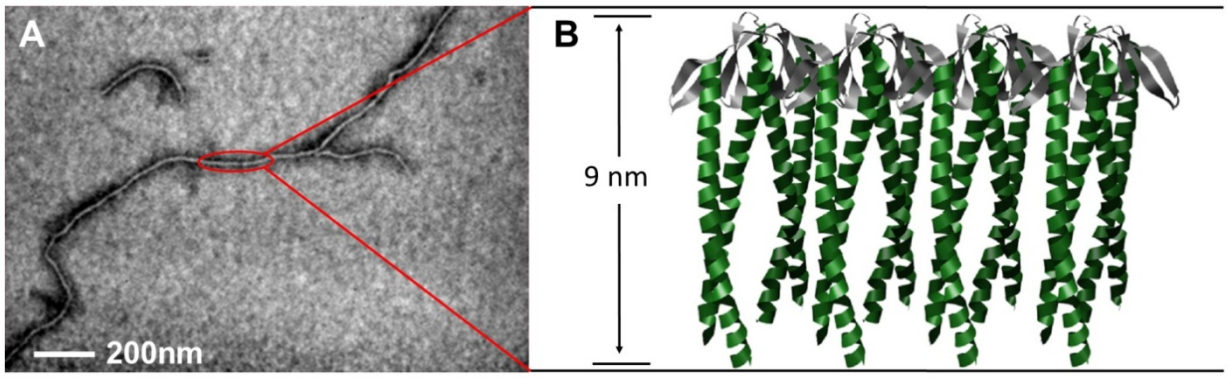

Figure 1. The structure of γ-PFD filaments. (**A**) Electron micrograph of a γ-PFD filament and (**B**) its quaternary structure. The packing of the subunits is perpendicular to the filament's longitudinal axis.

New 2D and 3D protein architectures of tailorable dimensions

We generated tunable two-way connectors that can connect two γ-PFD filaments together at a predefined angle. The design of a two-way connector was based on the genetic fusion of two complementary TERM monomers, resulting in a long coiled-coil linker region between the protein interaction (β-sheet) regions of the TERMs (Fig. 2A). The use of TERM ensures that filament growth can only occur in a single direction from each TERM interface within the two-way connector (Fig. 2B). When the two-way connector was combined with γ-PFD filaments we observed the creation of self-closing filaments, either circular or figure-eight in shape (Fig 3A). The coiled-coils of the TERMs have a predictable left-hand supercoil of 20° per seven amino acids, therefore it should be possible in future engineering of the two-way connector to change the projection angle of the filaments by the addition or subtraction of helical length of the two-way connector (Fig 2C). A three-way connector was created using a trimerization domain called foldon in fusion with TERM (Fig. 2D & E) as a method to link three γ-PFD filaments into a "pinwheel"-type structure (Fig. 2F). Subsequently, we demonstrated the ability of the three-way connector to trimerize into complexes that are highly stable to heat, and link together individual filaments (Fig. 3B). To confirm the structure of the three-way assembly, we were able to position gold nanoparticles at the center of the assembly (Fig 3C). This also highlights the potential application of the engineered connectors for nanoscale positioning of functional materials to create nanostructured materials (Objective 2 of this project).

The filaments of γ-PFD are highly variable in length, which results in the arms of the three-way pinwheel being non-uniform in length. Furthermore, because the pinwheels are capable of binding each other, it was not surprising that pinwheels often assemble together into random and

undefined assemblages. It was apparent that greater control over assembly of the connectors and γ-PFD filaments would be required for the creation of complex and higher-order 3D structures.

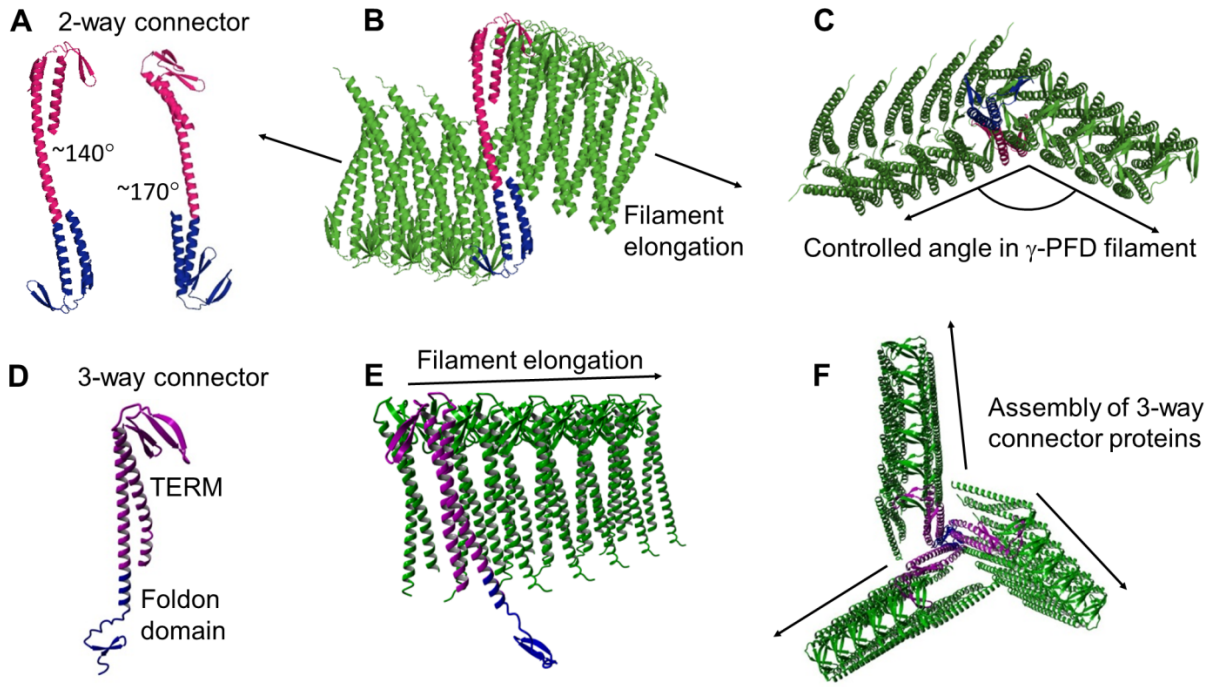

Figure 2. Engineering of two- and three-way connectors to link γ-PFD filaments and control their angles. (**A**) Structure of a two-way connector created by the fusion of two TERM monomers. Each connector is designed to impart a specific angle between the filaments, either 140° or 170° in this case. (**B**) Filaments of γ-PFD elongate from the connector in two directions (**C**) at a specific angle imparted by the orientation of the two TERM units in the connector. (**D**) TERM fused to the T4 fibritin foldon trimerization domain. (**E**) The γ-PFD units associate and elongate unidirectionally away from a TERM-foldon. (**F**) Pinwheel structure consisting of three γ-PFD filaments adjoined by TERM-foldon connectors.

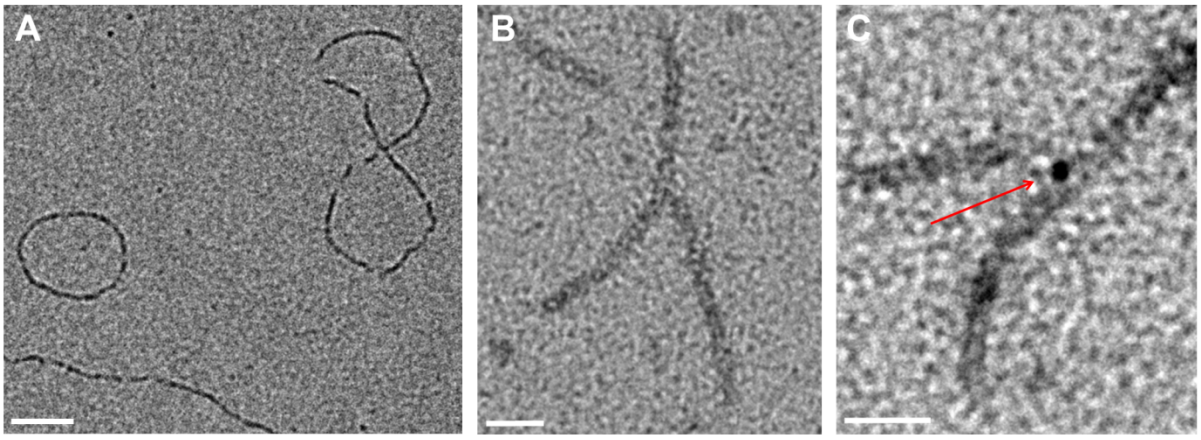

Figure 3. Transmission electron microscopy of γ-PFD filaments assembled with 2- and 3-way connectors. (**A**) Preliminary imaging of the 2-way connector mixed with γ-PFD showed self-closing structures either circular or "figure-eight" in shape. Scale bar = 200 nm (**B**) The 3-way connector was shown to assemble with three γ-PFD filaments and (**C**) its central location confirmed by the positioning of a gold nanoparticle bound specifically to the connector at the center of the pinwheel (arrows). Scale bars = 50 nm.

Design of tunable binding partners and control over filament length

Nature has the unprecedented capability to generate macromolecular assemblies from smaller structural units by utilizing highly complementary sites on these protein subunits. Designing such protein interfaces with defined arrangements, however, remains a challenging task due to the complexity and number of interactions involved. Only in the past decade has it proven possible to combine molecular understanding of such interactions with powerful computational methods to produce successful designs with reasonably high precision. Consequently, based on our initial success to incorporate a two- and three-way connector into the γ-PFD subunit we further expanded our functional toolbox with novel functionalities to gain greater control over assembly of the connectors and γ-PFD filaments.

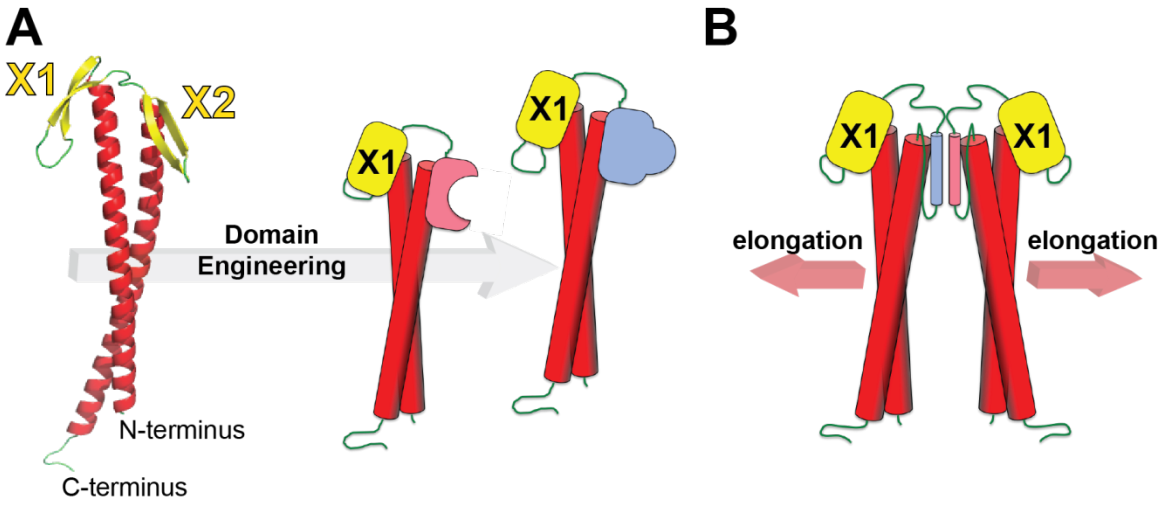

Figure 4: Engineering the X2-domain of γ-prefoldin to create tunable binding partners. (**A**) Previous studies with the wild type γ-prefoldin have shown that the X2-domain can be modified by rational design without affecting dimerization of the X1 domain. (**B**) Redesigning X2 to function as a "bait" (in pink) and "prey" (in blue) pair has generated complementary binding partners that can be used to connect two filaments of a desired length.

This was achieved by using the simple but well-understood structural elements of coiled-coils [6] to stimulate strong hydrophobic interactions between two self-associating helical bundles (Fig. 4A). As a consequence, two γ-PFD subunits containing opposing α-helical stretches (the so-called "bait" and "prey" sequences) form a tight heterodimeric coil-coiled bundle and elongate with the wild-type monomer to fibers of defined length (Fig. 4B). These bait and prey proteins were shown to specifically bind each other, and also function as capping proteins that terminate growth of the filaments. Therefore, the average length of the fibers could be controlled by adding the appropriate proportion of capping protein to γ-PFD. The bait-prey system is a complementary platform to our already existing connectors for the generation of more complex structures. We therefore added the bait sequence to the three-way connector (Fig 5) which enabled filaments that are capped with the prey protein to specifically bind to the three-way connector, thereby preventing uncontrolled assembly that occurred with the original three-way connectors. To gain control over filament length, we developed a strategy of capping filaments with the prey to limit

further growth and separating filaments based on length by size-exclusion chromatography. This methodology produces filaments of uniform length that are only able to bind the three-way connector containing a bait site (Fig 5).

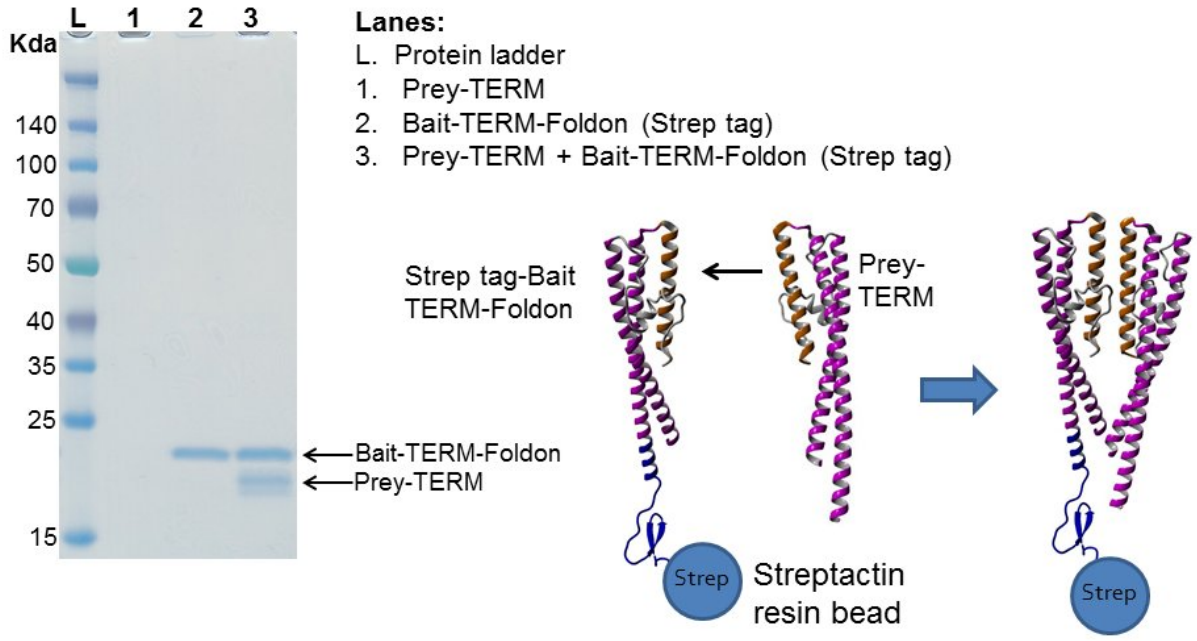

Figure 5: The engineered bait-prey proteins bind specifically to each other. A pull-down assay was used to show that the prey-TERM protein was able to bind to a three-way connector containing the bait sequence and a streptactin binding site. The bait-TERM-Foldon three-way connector in turn bound to streptactin resin, thereby pulling along the bound prey-TERM protein, which was then examined on an SDS-PAGE gel.

Assembly of higher-order structures

Ultimately our goal is to create self-assembling 3D structures using our two- and three-way connectors in conjunction with the bait-prey system to control the assembly process. We have attempted to assemble the three-way connectors into cube structures (Fig. 6). Firstly, the purified prey-TERM protein is mixed with wild-type γ-PFD to cap filaments and produce a distribution of filament sizes. Subsequently, the capped-filaments are applied to a Sephacryl S400 size-exclusion chromatography column and filaments of different length separated into filaments. These fractions that contain near-homologous sized filaments were incubated with the 3-way connector containing the bait domain. We are currently examining the resulting assemblages using TEM and cyro-EM to determine if cube-like structures have been created. Furthermore, as the length of the filament is controllable, we aim to produce protein cubes that vary in size and can be functionalized with nanoparticles (such as was achieved with the three-way connector, Fig. 3C).

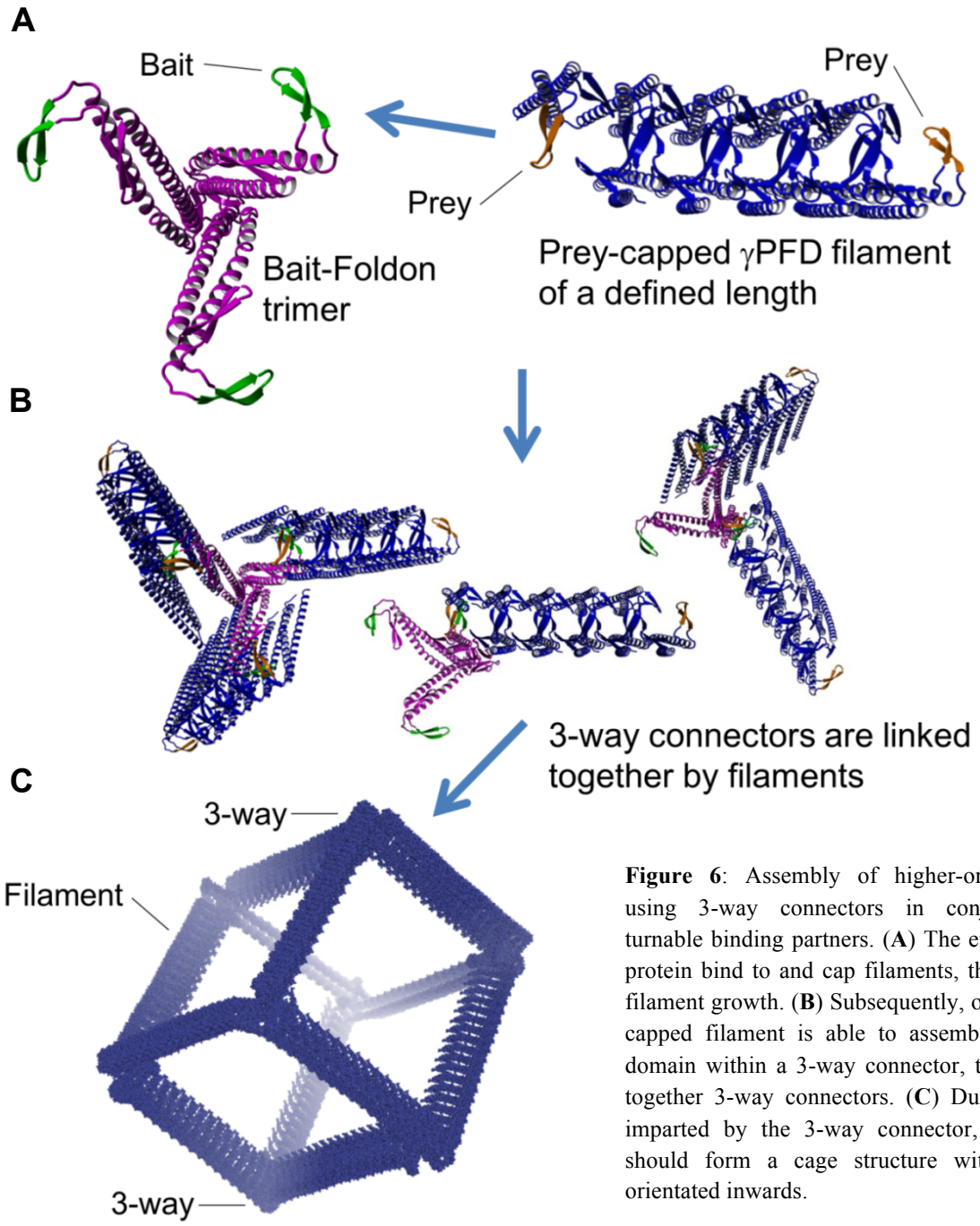

Figure 6: Assembly of higher-order structures using 3-way connectors in conjunction with turnable binding partners. (**A**) The engineered prey protein bind to and cap filaments, thereby limiting filament growth. (**B**) Subsequently, one end of each capped filament is able to assemble with a bait domain within a 3-way connector, thereby linking together 3-way connectors. (**C**) Due to the angle imparted by the 3-way connector, the assembly should form a cage structure with coiled-coils orientated inwards.

Expanding the versatility and processability of the γ-PFD

The development of protein templates that are stable under extreme conditions of heat or chemical denaturants will expand processing conditions and end-use applications for

biomaterials that require exceptional stability and robustness. We therefore attempted to stabilize the γ-PFD to even higher temperatures to exploit functional advantages, for example, in the nucleation of metals to form metallic nanowires along γ-PFD filaments. Using rational design principles, we hypothesized that increasing the hydrophobicity of the γ-PFD coiled-coil would result in increased packing and higher thermostability. To that end, we performed site-directed mutagenesis within the coiled-coil domain to replace polar and charged residues with more hydrophobic residues to yield fibrils with stability up to 103°C and increased length over the wild-type γ-PFD[3]. These enhanced thermostable mutants serve as excellent templates for the formation of metallic nanowires, where elevated temperatures facilitate metal nucleation. The incubation of the γ-PFD mutants with $(NH_4)_2PtCl_4$ at 100°C resulted in rapid nucleation and the production of a fibrous web of platinum nanowires (Fig. 7). Wild-type PFD, however, did not form nanowires due to the filaments clumping upon addition of the platinum salt, presumably due to the lower thermostability leading to shorter unstable filaments [3]. Ultrastable and malleable filaments that can function as architectural templates for nanomaterials, such as the enhanced γ-PFD filaments, will create new opportunities for material processing conditions and end-use applications that require exceptional stability and robustness. Such applications include the creation of scaffolds and cages to spatially arrange thermostable enzymes for high-temperature biocatalysis[7][8] and as biotemplates for fabrication and patterning of inorganic materials in the presence of chemical denaturant [9]. The enhanced γ-PFD filament created in this project may be particularly useful as a scaffold upon which to position hyperthermostable enzymes that have optimal activities at or above 100°C [8], which are temperatures beyond the stability of wild-type γ-PFD (objective 2 of this project). In addition, the temperature at which bio-nanomaterials are fabricated directly affects their rate of synthesis, as well as the resulting material's structure and physicochemical properties. Therefore, increasing the stability of a biotemplate such as γ-PFD will enable the creation of novel biomaterials currently unachievable using conventional proteins and standard processing conditions for biomolecules.

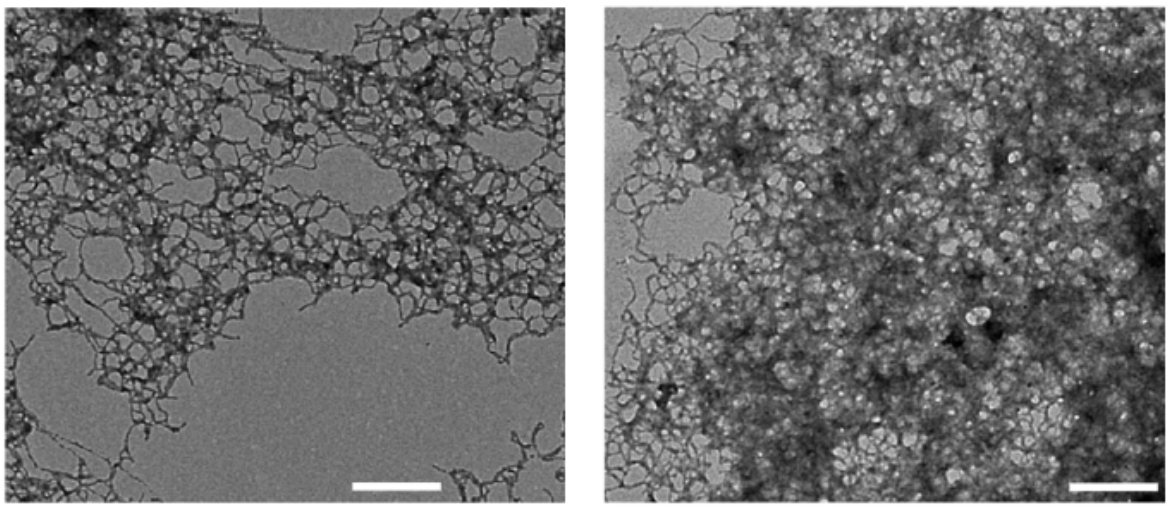

Figure 7. Transmission electron microscopy of platinum-decorated γ-PFD filaments that have been engineered to have higher thermostability (right) than wild-type PFD filaments (left) that were synthesized at 100°C (scale bars = 300 nm).

The minimal oligomer structure of γ-PFD for chaperone function

Previous studies have demonstrated that filaments of γ-PFD have similar chaperone function to the fully assembled hexameric (α/β) prefoldin [2]. The increased expression of γ-PFD in *M. jannaschii* exposed to extreme temperatures (95°) motivates further study of the functional chaperone mechanism of this protein. Furthermore, understanding how filament formation influences high-temperature chaperone activity or affects protein stability could assist in the future design of advanced materials with chaperone-like functions to improve the stability of functional proteins in extreme environments. It was unclear from previous studies whether the protein must assemble into a filament to have chaperone function, and if so, what influence does filament length have on chaperone ability. Using an established chaperone assay [2] we were able to demonstrate that the monomeric and dimeric assembly of γ-PFD does not have chaperone function compared to the wild-type filament. The methodology developed for the capping and separation of filaments based on length was used to fractionate filaments into specific sizes that varied from dimers to decamers. Subsequently, it was shown that the minimal oligomer length of the γ-PFD for chaperone activity was a hexamer, with no chaperone function observed for tetrameric γ-PFD assemblies. Importantly, this suggests that our 2D and 3D structures created with short 50-100 nm γ-PFD filaments will have full chaperone function (for example, the cube in figure 6 is composed of 60 nm filaments containing ~50 γ-PFD subunits each) and may have applications in stabilizing additional proteins, such as enzymes, fused to the 2D/3D structure.

Publication strategy

1. Our successful engineering of the γ-PFD to increase its thermal stability for the production of thermostable nanowires was published as an invited manuscript in a special issue "Nanobio versus Bionano" of *Biotechnology Journal* [3].
2. We are currently in the process of submitting a manuscript that defines the minimal length of γ-PFD that is required for chaperone function (titled "The minimal oligomeric structure required for the molecular chaperone function of the filamentous γ-prefoldin").
3. It is our intention to write a high-impact manuscript that details all the connector engineering (two- and three-way connectors) in conjunction with the bait-prey binding partners for the creation of a self-assembling cube structure. However, if the cube fails to assemble as expected from our protein modeling, we intend to instead write two separate manuscripts. The first manuscript will detail the engineering and assembly of the two-way connectors into self-closing loops, and the second manuscript will focus on the three-way connector and the bait-prey assembly to produce precise pinwheel structures that can be functionalized with nanoparticles (Fig 3C).
4. During the project we created a Forster resonance energy transfer (FRET) assay that enables the positioning of γ-PFD subunits within a filament to be examined and measured with angstrom resolution. We are currently using this assay as a method to control the spacing of γ-PFD

subunits in a filament, which will be written up for publication within the next few months. Ultimately, we aim to use this approach for the controlled spacing of enzymes in filaments or structures of γ-PFD for metabolic engineering applications.

5. We have an established a collaboration to use an engineered low pH-stable γ-PFD as a template for the nucleation of organic semi-conductors. We have supplied our collaborator, Professor Lisa Martin (Monash University, Australia), with the engineered protein and her laboratory is currently examining the templation of materials on the filament at low pH. Our experiments detailing the stability of the engineered low pH-stable PFD are complete and a manuscript will be submitted if we are successful in templating novel materials on the filament.

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